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ENVIRONMENT ONTARIO RESEARCH REPORT

KLEBSIELLA PNEUMONIAE MEMBRANE FILTRATION PROCEDURE

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DEPARTMENT OF MICROBIOLOGY

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ABSTRACT

Twelve different Klebsiella isolation media, mK2, mK4, mK5, mK6, mK6B, mCIK, mCIK2, UK1, UK2, mFC, mKleb, and mKleb2, were analyzed for their ability to specifically recover Klebsiella spp. from surface waters and sewers. Surface water samples and pure culture isolates were used to select the five best media. Results showed that mK2, mK4, mK5, mFC and mKleb provided similar recoveries of presumptive Klebsiella target colonies and target colony morphology was easy to distinguish from background colonies.

A total of 3190 presumptive Klebsiella target colonies were isolated from the five media and identified to the species level using the API 20E system. Approximately 93% of the presumptive target colonies on mFC, mKleb, mK4 and mK5 were identified as Klebsiella spp. Ninety-six percent of the presumptive target colonies on mK2 were identified as Klebsiella spp.

Performance characteristics were determined for the five different media according to ASTM specifications. The false positive error for mK4 was 0.058 while the other media had values approximating 0.015. Two media, mFC and mK5 had undetected target errors of 0; the undetected target error for mKleb, mK2 and mK4 was less than 0.06. The index of selectivity for mKleb was 0.53 while the other four media had an index of selectivity calculated to be approximately 0.64.

Accuracy values at 0 h for mFC, mK2 and mK4 averaged 114% compared to the reference medium mK base. The 24 h accuracy value calculated for mFC was 81.9% while mK2 and mK4 24 h accuracy values were 118.8% and 111.0%, respectively. The upper counting limit for mK2 was 115 colonies per filter while the upper counting limit for mFC and mK4 was 127 colonies.

Based on the performance characteristics and laboratory performance of the media, the medium most suitable for Klebsiella recovery is mK4.

RÉSUMÉ

Douze milieux d'isolement de Klebsiella, mK2, mK4, mK5, mK6, mK6B, mCIK, mCIK2, UK1, UK2, mFC, mKleb et mKleb2, ont été analysés afin d'évaluer dans quelle mesure ils parvenaient à récupérer les espèces Klebsiella des eaux superficielles et des eaux d'égout. Les cinq milieux les plus favorables ont été choisis à l'aide d'échantillons d'eaux superficielles et d'isolats purs. Les résultats ont montré que mK2, mK4, mK5, mFC et mKleb récupéraient les colonies cibles supposées de Klebsiella de manière analogue et que la morphologie des colonies cibles les rendaient faciles à distinguer des autres colonies.

Un total 3 190 colonies cibles supposées de Klebsiella ont été isolées des cinq milieux et identifiées à leur espèce à l'aide du système API 20E. Environ 93 % des colonies cibles supposées de mFC, mKleb, mK4 et mK5 ont été reconnues comme appartenant aux espèces Klebsiella. De même pour 96 % des colonies cibles de mK2.

Les caractéristiques de rendement des cinq différents milieux ont été déterminées selon les normes de l'ASTM. Le taux d'erreur faussement positive pour mK4 était de 0,058, tandis que les autres milieux avaient un taux d'environ 0,015. Deux milieux, mFC et mK5, avaient un taux d'erreur de détection de 0; le taux d'erreur de détection des milieux mKleb, mK2 et mK4 se situait en dessous de 0,06. L'indice de sélectivité de mKleb était de 0,53, alors que les quatre autres milieux avait un indice de sélectivité d'environ 0,64.

Les valeurs d'exactitude à 0 h pour les milieux mFC, mK2 et mK4 étaient en moyenne de 114 % par rapport à celles du milieu de référence mK. La valeur d'exactitude à 24 h calculée pour mFC était de 81,9 %, tandis que celle de mK2 et mK4 était de 118,8 % et 111,0 % respectivement. La numération limite de mK2 était de 115 colonies par filtre, alors que celle de mFC et de mK4 était de 127 colonies.

Si on se fie aux caractéristiques de rendement et d'efficacité des milieux en laboratoire, le milieu de récupération le plus approprié de Klebsiella est le mK4.

CONCLUSIONS

Accurate methods of water analysis must be developed in order to confidently assess the safety of our surface water supplies. Many factors can affect the interpretation of the results obtained from laboratories such as the poor recovery of target organisms, poor selectivity of media, and unknown reliability of media currently used.

The results of this study suggest that several different isolation media are unsuitable for determining levels of Klebsiella spp. in sewage and surface waters. Inappropriate media include UK1, UK2, mK6, mK6B, mCIK, mCIK2, and mKleb2.

The remaining media tested, mK2, mK4, mK5, mFC and mKleb all showed similar presumptive target colony recoveries with greater than 92% of these colonies identified as Klebsiella spp.

Approximately 15% of the identified Klebsiella spp. were K. pneumoniae sensu-stricto, and 18% were K. oxytoca. The prevalence of these two species found in the environment at these levels may be of concern due to their association with human and animal infections.

Statistical analysis of the media revealed that mK2 and mK4 are very similar with respect to all parameters. Based on laboratory performance, mK4 is the most suitable medium for determining levels of Klebsiella spp. in water samples. Colony crowding effects and problems regarding presumptive target colonies were minimized using this medium. In addition, an indication of the Klebsiella population was possible at 20 h post incubation, hence decreasing the elapsed time for any necessary repeat analysis.

RECOMMENDATIONS

1. This study suggests that routine membrane filtration analysis should use recovery medium mK4 when determining levels of *Klebsiella* spp. in surface waters and sewage effluents.

2. Epidemiological studies should be performed to determine if the presence of *Klebsiella pneumoniae* sensu-stricto at bathing beaches is a health hazard.

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INTRODUCTION

Bacteriological water quality guidelines are primarily based on the concentration of fecal coliforms (FC) or, more recently, E. coli (EC), depending on the jurisdiction involved. Ontario currently has a guideline of 100 FC per 100 mL but is considering switching to an EC based guideline to determine the safety of our natural waterways for recreational use.

The methods available to monitor surface waters have a good performance record for most types of samples. However, it has become apparent that analyses of pulp and paper effluent and waters significantly impacted by them yields inaccurate results when standard FC or EC procedures are utilized. This problem has been created by the presence of large concentrations of Klebsiella spp. in these wastes (Niemela and Vaatanen, 1982; Dufour and Cabelli, 1976) that grow as target colonies on the FC/EC media currently in use.

Klebsiella are ubiquitous in nature (Bagley, 1985) primarily due to the fact that water soluble compounds, such as sugars liberated from ground pulp or from vegetable surfaces, promote the growth of this microorganism (Knittel et al, 1977; Caplenas et al, 1981). Consequently, high concentrations of this bacterium may lead to a false impression of fecal pollution. It thus becomes important to accurately identify the presence of Klebsiella spp. and the respective level of the organism to aid in the interpretation of water quality data.

Although Klebsiella bacteria are usually associated with pulp and paper effluent, vegetable produce and living trees (Dufour and Cabelli, 1976; Duncan and Razzell, 1972; Huntley et al, 1976), this microorganism has been frequently isolated from human and animal infections (Bagley and Seidler,

1977; Braman et al, 1973; Selden et al, 1971). In addition, Bagley and Seidler (1977) have reported that Klebsiella from diverse environmental origins, regardless of biotype, are potentially as pathogenic as Klebsiella of known clinical origins.

The Klebsiella genus currently consists of K. pneumoniae subspecies pneumoniae, K. oxytoca, K. planticola, K. terrigena, K. pneumoniae subspecies rhinoscheromatis and K. pneumoniae subspecies ozaenae. Two different biotypes described by Naemura and Seidler (1978) are K. pneumoniae sensu-stricto and K. pneumoniae non sensu-stricto. The biotype sensu-stricto has been most frequently associated with human/animal clinical origins (Naemura and Seidler, 1978); however, more recently K. oxytoca and K. pneumoniae non sensu-stricto have been encountered in a variety of human and animal infections (Bagley, 1985).

The increasing interest in Klebsiella spp. is not only due to the interference problems regarding current water quality guidelines, but also due to the fact that these microorganisms may be more pathogenic than other environmentally-derived gram-negative organisms (Bagley and Seidler, 1978).

Several different media have been proposed for the recovery of Klebsiella pneumoniae, including MacConkey-inositol potassium tellurite agar (Tomas et al 1986), m-K medium (Dufour and Lupo, 1977), double violet agar (Campbell and Roth, 1975), modified mFC medium (Stramer, 1976), and mKleb medium (Geldrich and Rice, 1987). Methyl violet 2B, phenol red, carbenicillin, bile salts, uric acid, inositol and differing nutrient base compositions are among the agents most often altered in the development of new recovery media in order to achieve optimum selectivity and recovery of the microorganism in question.

Reports have been published regarding the efficiency of many different Klebsiella isolation media (Geldrich and Rice, 1987; Tomas et al, 1986; Dutka et al, 1987); however, none have been statistically evaluated for use in Ontario with environmental samples. In addition, there is a paucity of information on the recovery rates of the various species and biotypes of Klebsiella by any of these methods.

Objectives

The objectives of this study were to:

1) Develop a protocol for the enumeration of Klebsiella spp. from environmental samples.

2) Determine the ability of experimental methods to isolate Klebsiella pneumoniae sensu-stricto.

3) Assess and document the performance characteristics of the method of choice.

MATERIALS AND METHODS

Sample Collection

Surface water samples were collected in bottles containing sodium thiosulphate (Appendix) which were kept on ice during transport to the laboratory. St. Catharines beaches, Humber and Don Rivers, Etobicoke Creek and Toronto area sanitary sewer outfalls were selected as the sampling sites. Samples were analysed within 24 hours.

Media Screening Investigation

Appropriate dilutions of each water sample were made in phosphate buffer (Standard Methods, 1987; Appendix) and the dilutions membrane filtered using standard techniques (APHA, 1985). Aliquots and/or dilutions ranging from 50 mLs to 10^{-5} mLs, were filtered through sterile 0.45um porosity membranes (GN-6, Gelman Sciences) and subsequently placed on the isolation media. All plates were incubated inverted at 35°C. Media used in the analysis were mK2, mK4, mK5, mK6, mK6B, modified UK1 (UK1), modified UK2 (UK2), modified mFC (mFC), mCIK, mCIK2, mKleb and mKleb2. The former 7 media were incubated for 48 h while the latter 5 were incubated for 24 h. After the specified incubation period, counts of target and non-target colonies were made.

Sterile dilution blanks (Appendix) were spiked with cultures of Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli, and membrane filtered as described by APHA (1985). Filters were placed on the test media and plates incubated. Presence or absence of growth as well as colony morphology were noted after the incubation period.

Target and non-target colonies were picked from each medium and streaked onto BHI agar (Difco) for purity. Isolates were identified using API-20E strips (Analytab Products) and/or standard biochemical tests.

Isolation of Bacterial Cultures

Five different isolation media were selected for isolating potential Klebsiella pneumoniae sensu-stricto cultures. Water samples obtained from St. Catharines were membrane filtered in quintiplicate and one filter from each dilution placed on mK2, mK4, mK5, mKleb and mFC. After the appropriate incubation period, counts of target and background colonies were made. Those filters which had between 10 and 100 target colonies were retained for bacterial isolation.

Target colonies were picked from each filter using a sterile wire, streaked on BHI agar plates and incubated at 35°C for 18 - 24 h. To ensure purity, cultures were passed on BHI agar at least twice.

Cultures were prepared for long term storage by pouring 2 mL of glycerol solution (Appendix) over the freshly grown pure culture (24 h) and the culture mixed into the glycerol solution. Sterile filter strips (Whatman #5) placed in freezer bags were moistened with a sample of the bacterial suspension. Freezer bags were heat sealed and placed at -70°C.

Approximately 10 target colonies were picked from each selected filter. A total of 3190 isolates were isolated and frozen in duplicate.

Isolate Recovery

The preserved cultures were thawed at room temperature, the moistened filter paper removed from the pouch and placed on BHI agar plates. A sterile inoculating loop was used to streak outward from the filter paper

to obtain isolated colonies. Plates were incubated at 35°C for 18 - 24 h. One isolated colony picked from overnight growth of the initial plate was restreaked on BHI agar for isolated colonies.

Gram Reaction

Isolated colonies were examined for colonial morphology on BHI agar. A few isolated colonies were picked from an overnight culture and subjected to the Gram reaction as described by Fluharty and Packard (1967). Each isolate was mixed with a drop of 3% KOH solution on a slide and examined for suspension consistancy. Gram-positive organisms do not form a gel when mixed with the 3% KOH soution. Gram-negative organisms form a thick gel when mixed with the KOH solution.

Oxidase Production

A 1% solution of N,N,N,N-tetramethyl-p-phenylene diamine (Appendix) was impregnated on clean Whatman #5 filter paper discs and allowed to dry. A sample of the freshly grown culture was picked with a sterile toothpick and rubbed into the treated filter disc. A purple coloration on the filter paper was considered as a positive reaction.

Bacterial Identification

A sample of the isolate was mixed in a test tube containing 5 mL of sterile 0.85% saline. This suspension was inoculated into API-20E strips according to manufacturer's directions. After incubation, reagents were added to the cupuelles and the reactions recorded. Organisms were identified according the profile number generated from the tests.

Sensu-Stricto Testing

Isolates which were identified as Klebsiella pneumoniae, based on the API testing, were subjected to further biochemical testing in order to designate them as sensu-stricto or non sensu-stricto. Biochemical tests included proline utilization, sorbose fermentation, growth and gas production in EC broth and growth in nutrient broth at 10°C.

Overnight cultures of K. pneumoniae were inoculated into 5 mL aliquots of nutrient broth and incubated at 35°C for 18 h. A 100 uL sample of the broth culture was inoculated into hydroxy-l-proline broth (Appendix) and incubated at 35°C for 72 h. This test was regarded positive if growth of the culture was noted in the tube. Sorbose testing was performed by inoculating a small portion of the broth culture into 5 mL of sorbose broth (Appendix). After incubation at 35°C for 72 h, the presence of a yellow coloration of the broth, denoting acid production, was considered positive while red or orange colortion was considered negative.

EC broth (Difco) was inoculated with a portion of the overnight broth culture and subsequently overlaid with a suspension of sterile 3% molten agar. After the agar had solidified, EC tubes were incubated at 44.5°C for 3 days. Tubes were observed for the presence of growth (turbidity) and the production of gas (displacement of the agar plug), both considered as positive results.

To assess growth of the K. pneumoniae isolates at 10°C the following procedure was performed. A 10 uL sample of the overnight culture was added to 10 mL of sterile nutrient broth (Difco) and immediately incubated at 10°C for 3 days. Presence of growth was assessed by determining the absorbance at 650 nm. A reading of > 0.02 was considered positive. Isolates were considered as sensu-stricto according to the reactions listed in Table 1.

TABLE 1: Selected Biochemical Reactions of Klebsiella pneumoniae sensu-stricto.

EC Growth at 45°C	EC GAS	Growth at 10°C	Proline	Sorbose
+	+	-	-	-
+	+	-	+	-
+	+	-	-	+

Combinations of reactions different than those listed here designated the isolate as non sensu-stricto.

Media Performance Characteristics:

Selectivity and Specificity

Five different media, mFC, mKleb, mk2, mK4 and mK5 were chosen to determine which of the five media was the best at performance characteristics of selectivity and specificity.

Five fold dilutions of water samples obtained from beaches, rivers and sanitary sewer outfalls were membrane filtered in triplicate and placed on the above mentioned media as previously described. After the designated incubation period, counts of target and non-target colonies were made.

All of the colonies from plates which contained at least 30 presumptative target colonies were picked and streaked on to BHI agar plates. Incubation was for 24 h at 35°C. Pure colonies of each culture were subjected to the Gram-reaction and oxidase production tests. Those isolates which were found to be Gram-negative and oxidase-negative were identified to the species level using API 20-E strips.

Accuracy

Water samples from Lake Ontario were filter sterilized and dispensed in 99 mL aliquots into sterile sample bottles. Each sample was seeded with one mL of an overnight nutrient broth culture of either Klebsiella pneumoniae, Klebsiella pneumoniae sensu-stricto, or Klebsiella oxytoca. All of the cultures used in this experiment were environmental isolates.

Appropriate dilutions of the seeded water sample were made in phosphate solution and subsequently membrane filtered using 0.45 um filters. Five replicate filters from each dilution were placed on mKBase (Appendix), mK2, mFC and mK5. A total of two different isolates of each Klebsiella spp. were

used in this analysis. After incubation, colony forming units were recorded.

The original dilution (one mL of overnight broth culture diluted in 99 mL of membrane sterilized lake water) was stored at 10°C for 24 h. After this stress period, the seeded water samples were diluted accordingly and membrane filtered. Filters were placed on the 4 media in quintuplicate. Plates were incubated as necessary.

Counting Range Analysis

A total of sixty-one water samples collected from the St. Catharines beaches, Humber River and sanitary sewer outfalls were diluted in 5 fold increments (i.e. 3 mL of sample in 12 mL of dilution buffer). Dilutions were membrane filtered and filters from each dilution placed on mK2, mK4 and mFC in triplicate. After the appropriate incubation period, colony forming units were assessed. Only those filters which had a mean colony count of greater than eight colonies were considered in the analysis.

RESULTS

Media Screening Results

Several different screening tests were performed on the media prior to recovery of bacterial isolates. The first screening test of the study involved nine different media: mK2, mK4, mK5, mK6, mKleb, mFC, mCIK, UK1 and UK2. Surface water samples obtained from the Don and Humber Rivers, one sanitary sewer sample and one water sample seeded with Klebsiella pneumoniae were subjected to membrane filtration.

Description of target and background colonies for each medium used in this study are listed in Table 2. These guidelines were used in enumeration of potential Klebsiella pneumoniae colonies. Target and background colonies were identified to confirm speciation of the isolates.

Table 3 lists colony forming units (CFU's) of Klebsiella pneumoniae isolates on the different media. Similar target colony counts were obtained using mK2, mK4, mK5, mKleb, mCIK and mFC. The number of target colonies observed on UK1 and UK2 were approximately 100 fold greater than counts obtained using other media. The predominate colony type on UK1 and UK2 were generally < 1 mm in size and rose-white in color as compared to the typical target colony morphology description (Table 2). Target colonies on the urea based media were identified as 50% Klebsiella spp. and 50% non-Klebsiella spp.

Four media, UK1, UK2, mCIK and mK6, were unable to recover the environmental isolate of Klebsiella pneumoniae from the seeded water sample (Table 3). The isolation medium mK6 was unable to support the growth of any type of bacteria.

Table 2: Target and Background Colony Morphology on Media Used to Isolate *Klebsiella* spp.

Medium	Target Colony Description	Background Colony Description
mK2	1 - 3 mm smooth yellow mucoid	-orange 1 - 3 mm colonies -pinpoint white-green colonies
mK4	1 - 3 mm smooth mucoid colonies Colonies have blue coloration and a blue halo.	-small (<0.5mm) white colonies without a blue halo -brown to green colonies
mK5	1 - 3 mm smooth mucoid colonies Colonies are black in color surrounded by a blue halo.	-colonies without a blue halo or colonies that have a blue halo but no black coloration
mK6	3 - 5 mm lavender colored colonies, mucoid	-dark purple colonies 1 - 3 mm
mKleb	2 mm greyish blue colonies or blue centered with a grey edge.	-pink, yellow or orange colonies 1 - 3 mm in size
mFC	same as mKleb	-same as mKleb
mCIK	Chocolate brown up to 2 mm in size. Soft colony.	-dark brown/black to tan in color colonies, 1-3mm in size
mCIK2	Pink to red colonies approx. 2 mm in size.	-same as for mCIK
UK1	Pink tinted colonies, soft and very mucoid. Usually <1mm.	-white to clear colored colonies up to 2mm in size
UK2	Same as listed for UK1.	
mKleb2	2mm black centered colonies with a blue halo. Some *Klebsiella* spp. colonies appeared as black pinpoint colonies with a blue halo.	-brown pinpoint and white-yellow opaque colonies
mK6B	3 - 5 mm lavender colonies very mucoid in consistancy.	-same as mK6

Table 3: Preliminary Trial #1; Total Colony Forming Units of *Klebsiella pneumoniae* on Different Media

Medium	WATER SAMPLE				
	Don River A	Don River B	Humber	Sewer	*K. pneumoniae*
mK2	5.0×10^3	2.5×10^3	5.2×10^2	3.4×10^3	3.6×10^9
mK4	6.0×10^3	9.0×10^2	2.6×10^3	7.2×10^3	2.2×10^9
mK5	6.2×10^3	3.0×10^3	8.2×10^2	7.0×10^3	2.7×10^9
mK6	0	0	0	0	0
mKleb	1.0×10^4	1.6×10^4	9.0×10^3	5.0×10^2	3.9×10^9
UK1	3.6×10^5	5.1×10^4	4.2×10^4	5.9×10^4	0
UK2	2.8×10^5	1.7×10^4	5.6×10^4	2.6×10^4	0
mFC	8.8×10^3	1.1×10^4	5.0×10^2	3.8×10^3	3.0×10^9
mCIK	1.1×10^4	TNTC	1.4×10^4	TNTC	0

Colony forming units per 100 mL sample.

TNTC = Too numerous to count.

Target colony identification on mCIK was difficult as very few colonies displayed typical target colony morphology as described by Tomas and coworkers (1986). Different colony types were picked from this isolation medium and identified. Colonies which were 2 mm grey in color were identified as Klebsiella pneumoniae while grey-brown 1-3 mm colonies were found to be Pseudomonas spp. (Table 4).

The second screening test involved isolation media mK2, mK4, mK5, mK6B mKleb, mFC and mCIK2. Three water samples from the St. Catharines beaches as well as seeded water samples of E. coli and Pseudomonas aeruginosa were membrane filtered and filters placed on the above mentioned media. Results from this analysis are listed in Table 5.

Total colony forming units using the above mentioned media were approximately equal except for mCIK2. This latter medium was found to produce target colony counts approximately 100 fold greater than counts observed on other media. Target colonies observed on mCIK were not found to conform with expected results (Tables 3 and 4). Colony morphology versus speciation showed that expected target colonies of Klebsiella pneumoniae were not present. Other colony types that were picked and identified were found not to be Klebsiella spp. (Table 4).

Colony forming units using the water sample seeded with Pseudomonas aeruginosa were observed on mCIK and mK2. These colonies on mCIK were brown to brown-green in color and 1 - 3 mm in size. Colonies of Pseudomonas aeruginosa on mK2 varied from yellow to white pinpoint colonies.

None of the media tested up to this point in the analyses recovered E. coli from the seeded water samples even at concentrations of 10^7 per 100 mL of sample.

TABLE 4: Identification of Selected Isolates on Media Used in Screening Test #1.

Medium	Colony Description	Identification
mCIK	1 mm brown colonies	*Pseudomonas* spp.
mCIK	2 mm grey colonies	*Pseudomonas* spp.
mCIK	2 - 3 mm flat grey colonies	*Klebsiella* spp.
mCIK	2 mm light brown colonies	*Klebsiella* spp.
mKleb	2 mm grey with blue centre	*Klebsiella pneumoniae*
mKleb	2 mm grey colony	*Enterobacter* spp.
mK4	2 mm blue halo colony	*Klebsiella pneumoniae*
mK4	pinpoint blue halo colony	*Klebsiella pneumoniae*
mK4	dark blue pinpoint colony	*Pseudomonas* spp.
UK1	pinpoint rose colony	*Pseudomonas* spp.
UK1	pinpoint white colony	*Pseudomonas* spp.
UK1	1 mm rose colony	*Klebsiella* spp.
UK2	pinpoint rose colony	*Pseudomonas* spp.
UK2	1 mm sticky white colony	*Klebsiella pneumoniae*
UK2	1 mm rose colony	*Klebsiella* spp.

TABLE 5: Screening Test #2; Colony Forming Units of *Klebsiella pneumoniae* on Selected and Modified Media.

Medium	Water Sample				
	9359*	9358	9361	*E. coli*	*Ps. aeruginosa*
mK2	2.7×10^6	8.4×10^5	1.2×10^6	0	TNTC
mK4	1.7×10^6	9.3×10^5	1.1×10^6	0	0
mK5	2.3×10^6	7.5×10^5	9.8×10^5	0	0
mK6B	1.2×10^6	4.8×10^5	9.5×10^5	0	0
mKleb	2.5×10^6	7.1×10^5	1.7×10^6	0	0
mFC	2.1×10^6	8.5×10^5	1.3×10^6	0	0
mCIK2	9.8×10^7	6.6×10^7	8.7×10^7	0	TNTC

* Refers to specific samples obtained from St. Catharines.

TNTC = Too numerous to count.

Identification of target versus background colonies was difficult using the medium mK6B due to the wide variety of colony morphologies, sizes, textures and differences in coloration.

Six water samples obtained from the St. Catharines area were used in the third media screening analysis. In addition, spiked water samples of Klebsiella pneumoniae and Pseudomonas aeruginosa were subjected to the same membrane filtration analysis. Results of this analysis are presented in Table 6.

Target colony counts on mK2, mK4, mK5, mKleb and mFC were found to be of the same magnitude, generally less than one half log unit in difference.

The medium mKleb2 supported the growth of three different types of bacterial colonies: 1) small black centered colonies with a blue halo, 2) 3mm black colonies with a blue halo, and 3) brown pinpoint or white opaque colonies. Colony types 1 and 2 were identified as Klebsiella pneumoniae. Taking this information into consideration, target colony counts using mKleb2 were found to be approximately 1 log unit lower in comparison to the other media used in this analysis.

The stock suspension of Klebsiella pneumoniae was unable to be recovered using mCIK and mKleb2. In addition, problems in quality control of media UK1, UK2, mK6, mKleb2, mCIK and mCIK2 were encountered during all of the analyses. These media were found to either not support the growth of several different environmental isolates of Klebsiella pneumoniae or exhibit very poor growth of K. pneumoniae in pure cultures.

Table 6 also lists the levels of background colonies observed on some media. Samples D and F had background levels at or near the target colony levels on mCIK and mKleb2. Background levels on the other media did not impair target colony enumeration from samples D and F.

TABLE 6: Screening Test #3: Total Colony Forming Units of Klebsiella spp. on Various Isolation Media.

Medium	Water Sample A	B	C	D	E	F	Klebsiella
mK2	8.0×10^4*	1.5×10^6	3.3×10^6	5.6×10^2	9.3×10^5	9.8×10^3	2.1×10^{10}
mK4	1.9×10^5	1.6×10^6	2.1×10^6	2.1×10^2	9.3×10^5	9.0×10^3	2.1×10^{10}
mK5	6.0×10^4	1.2×10^6	1.8×10^6	7.3×10^2	7.3×10^5	7.4×10^3	1.7×10^{10}
mKleb	1.3×10^5	1.7×10^6	2.6×10^6	3.6×10^2	1.1×10^6	6.9×10^3	2.0×10^{10}
mKleb2	5.0×10^4	3.2×10^5	5.1×10^5	3.1×10^2	1.8×10^5	2.9×10^3	-
mFC	6.0×10^4	9.0×10^5	2.7×10^6	1.8×10^3	6.8×10^5	8.1×10^3	2.1×10^{10}
mCIK	1.0×10^4	2.2×10^5	3.6×10^5	8.0×10^2	1.4×10^5	1.9×10^3	-
Background Levels							
mCIK	7.0×10^4	4.0×10^3	-	1.2×10^3	-	2.9×10^4	-
mKleb2	-	-	-	$>3.0 \times 10^2$	-	$>3.0 \times 10^3$	-

* Colony forming units per 100 mL sample.

Identification of Target Organisms

Table 7 lists the percent frequency of identified target microorganisms recovered from environmental and sewer water samples. Approximately 600 target colonies were recovered from each medium and identified to the species level. Those that were identified as Klebsiella pneumoniae were subjected to further tests to determine if they were sensu-stricto or non sensu-stricto.

All of the media used in this analysis were similar in the ability to recover Klebsiella spp. Target colonies were correctly identified as Klebsiella spp. at a rate of 96% using the medium mK2. On average, 93% of the target colonies present on mK4, mK5, mFC and mKleb were identified as Klebsiella spp.

Further analysis of the particular species of Klebsiella isolated showed that correct target colony classification of K. pneumoniae using mK2, occurred at a rate of 81%. Target colonies isolated from mFC were identified as K. pneumoniae 74% of the time. Target colonies on mK4, mK5 and mKleb, were positively identified as Klebsiella pneumoniae at a rate of approximately 77%.

The organism most commonly identified as forming a false target colony was Enterobacter spp. Other groups of microorganisms including Pseudomonas, Aeromonas and Vibrio spp. occasionally formed presumptive target colonies.

Klebsiella oxytoca was recovered at similar rates using all five media. The frequency of target colony classification of K. oxytoca ranged from 19% using mFC, to 16% using the four other media.

The specific sensu-stricto biotype of the Klebsiella pneumoniae identified isolates ranged from 9 to 18% on mK5 and mK2 respectively. The

recovery of K. pneumoniae sensu-stricto using mK4, mFC and mKleb averaged 16%. In comparison, the isolation of other K. pneumoniae biotypes using the five media ranged from 57% using mFC to 69% using mK5 (Table 7).

Specificity and Selectivity

Two water samples collected from St. Catharines were membrane filtered in triplicate and the filters placed on mK2, mK4, mK5, mFC and mKleb. After the incubation period, counts of target and non-target colonies were made. All of the colonies from plates with mean target colony counts between 30 and 100 were picked and identified in order to determine the specificity and selectivity criteria of the test media.

Indicies of specificity were determined using the equations:

$$\text{False positive error} = \frac{\text{False positive colonies}}{\text{Presumptive target colonies examined}}$$

$$\text{Undetected target error} = \frac{\text{Undetected target colonies}}{\text{Presumptive target colonies} - \text{False positive colonies} - \text{Undetected target colonies}}$$

The index of selectivity was determined using the formula:

$$\text{Index of selectivity} = \frac{\text{Presumptive colonies examined}}{\text{Total Countable Colonies}}$$

All of the calculations were performed according to section D3870 in the "Annual Book of ASTM Standards" (1986).

Table 8 lists the specificity and selectivity values of the 5 media. The false positive error was approximately the same using mK2, mFC, mKleb and mK5, a value found to be 0.014. The false positive error for mK4 was determined to be 0.058. The undetected target error was 0 for mFC and

TABLE 7: Percent Frequency of Identified Bacteria Using Specified Media

Isolate Identification	Isolation Medium				
	mK2	mK4	mK5	mKleb	mFC
Klebsiella spp.#	96	93	93	92	93
*Klebsiella pneumoniae**	81	77	78	76	74
Klebsiella pneumoniae non sensu-stricto	63	61	69	62	57
Klebsiella oxytoca	15	16	15	16	19
Klebsiella pneumoniae sensu-stricto	18	16	9	14	17
Enterobacter spp.	3	6	6	4	1
Other	1	1	1	4	6

Klebsiella spp. refers to *K. pneumoniae* and *K. oxytoca* groups.

* Refers to both *K. pneumoniae* sensu-stricto and *K. pneumoniae* non sensu-stricto.

TABLE 8: Specificity and Selectivity Values Calculated For Klebsiella Isolation Media

	Colony Forming Units on Various Media				
Colony Type	mK2	mK4	mK5	mFC	mKleb
Presumptive Target	67	69	73	67	76
Presumptive Non Target	37	41	40	37	66
False Positive Target	1	4	1	1	1
Undetected Target	3	1	0	0	5
	Selectivity Parameters				
False Positive Error	0.015	0.058	0.014	0.015	0.013
Undetected Target Error	0.0435	0.0152	0	0	0.0625
Index of Selectivity	0.64	0.62	0.64	0.64	0.53

mK5; 0.015 for mK4; 0.0435 for mK2 and 0.0625 for mKleb. Index of selectivity was calculated to be 0.64 for mK2, mFC and mK5, 0.62 for mK4 and 0.53 for mKleb.

Accuracy

The accuracy of the media was determined by filtering a water sample at time = 0 h and again after the sample had been stressed for 24 h at 10°C. The test media mK2, mK4 and mFC were tested against mK basal medium.

Accuracy determinations were made according to ASTM document D3870 (1986) using the equations:

$$\text{Accuracy (0 h)} = \frac{\text{Test medium count}}{\text{Reference medium count}} \times 100\%$$

$$\text{Accuracy (24 h)} = \frac{\text{Test medium count at 24 h}}{\text{Reference medium count at 24 h}} \times 100\%$$

Accuracy levels determined using six different environmental strains of Klebsiella spp. are listed in Table 9. All three media had similar accuracy levels at 0 h averaging approximately 114%. The accuracy values of the media were found to differ at 24 h by 30 percentage points. The medium mFC had the lowest 24 h accuracy level followed by mK4 and mK2. Values were 81.9%, 111.0% and 118.8% respectively.

Upper Limit of Counting Range

Sixty-one surface water samples from beaches, pulp and paper effluents, sanitary sewers and natural rivers were membrane filtered using 5 fold dilutions. Target colony densities were determined using neighboring dilutions with the lower counting range averaging greater than 8 colonies

per filter. The upper counting range was determined using the u test formula given by Hald (1960):

$$u = \frac{(5 \times LC) - HC - 1}{(5 \times LC) + HC} > 1.96$$

where LC refers to the lower count and HC, the higher count of bacterial colonies observed on the filters ($n > 8$).

The upper counting range for mFC, mK2 and mK4 are listed in Tables 10, 11 and 12 respectively. The medium mK2 had the lowest upper counting range, reported at 115 colonies. The media mK4 and mFC had an upper counting range of 127 colonies per filter.

TABLE 9: Accuracy Values as Determined Using Three Different Klebsiella Isolation Media

Klebsiella Strain	Colony Counts as Observed on Specified Media							
	mFC		mK2		mK4		Basal Medium	
	0h	24h	0h	24h	0h	24h	0h	24h
720	58	100	61	180	58	150	22	170
514	35	81	39	120	39	120	40	120
1104	34	64	38	78	25	78	22	28
499	25	7.6	21	7.3	22	7.4	72	6.1
1102	52	35	47	34	62	37	72	30
786	13	26	14	36	13	33	14	29
Average Recovery	36.2	52.3	36.7	75.9	36.5	70.9	31.8	63.9
Accuracy 0h	113.6%		115.2%		114.7%		-	
Accuracy 24h	81.9%		118.8%		111.0%		-	

TABLE 10: Upper Counting Range Results Using

Isolation Medium mFC

LC	HC	LC	HC	LC	HC	LC	HC
8	to 31.3	11	to 52	16.7	to 81.3	26.7	to 115
8	to 38.7	11	to 65	17.3	to 78.7	28	to 146
8	to 41.7	11.3	to 47.7	17.7	to 72.3	30.3	to 142
8	to 55.6	12	to 51.3	17.7	to 93.3	30.7	to 138
8.7	to 48.3	12	to 74.7	18	to 79.7	31.3	to 129.3
9	to 51.7	12.7	to 58.3	18.3	to 89.7	31.7	to 131.7
9.7	to 42.3	12.7	to 59.7	18.3	to 99.7	32.3	to 140
10	to 49	12.7	to 67.3	18.3	to 102.7	33.3	to 127.3*
10.3	to 47.3	14	to 65.3	19	to 107.7	33.7	to 125*
10.3	to 53.7	15	to 68.7	20	to 99.3	34.3	to 132.3*
10.3	to 55.7	15.3	to 76.3	20.3	to 92.3	38.7	to 105*
10.3	to 59.7	16	to 81.3	21	to 107.7	47	to 150*
10.3	to 60	16.3	to 83.3	23	to 123.3		
10.7	to 58.7	16.3	to 83.3	23.3	to 113		
10.7	to 60	16.7	to 72	24.3	to 103		
10.7	to 60.7	16.7	to 73	24.7	to 123		

* The u test values are greater than 1.96 and therefore the expected 5 x LC and observed HC are not members of the same distribution of means. The upper counting range for this medium is 127 colonies.

TABLE 11: Upper Counting Range Results Using

Using Isolation Medium mK2

LC	HC	LC	HC	LC	HC	LC	HC
8	to 40.3	10.3	to 49.3	15.6	to 64.7	23	to 108.3
8.3	to 33	10.7	to 38.7	16	to 91.7	23.3	to 97.3
8.3	to 35.7	10.7	to 39.7	16.3	to 85	24	to 98.3
8.3	to 36.3	10.7	to 46.3	16.7	to 66.7	24.3	to 107
8.3	to 37.3	11	to 42.3	17	to 73	24.3	to 109.3
8.3	to 42	11	to 52.3	17.3	to 72.7	24.3	to 121.3
8.3	to 44	11	to 69.7	17.3	to 79.3	25	to 124.7
8.3	to 52.7	12	to 64.3	17.3	to 97.7	26.3	to 113
9.3	to 46.7	13	to 59.7	19.3	to 81	29	to 130.7
9.3	to 53.3	13.3	to 57	19.7	to 90.3	31	to 153.7
9.3	to 54	13.7	to 58	20	to 82.7	31	to 115*
9.7	to 34	14	to 60.3	20.7	to 99.7	34	to 130*
9.7	to 34	14.3	to 64	20.7	to 113	34.3	to 125.6*
10	to 36	15.3	to 60	21.3	to 92.7		
10	to 64.3	15.3	to 65.3	22.3	to 119		

* The u test values are greater than 1.96 and therefore the expected 5 x LC and observed HC counts are not members of the same distribution of means. The upper limit of the counting range for mK2 would be 115 colonies.

TABLE 12: Upper Counting Range Results Using the Isolation Medium mK4

LC	HC	LC	HC	LC	HC	LC	HC
8	to 55	13	to 64.3	18	to 89.3	25	to 115.7
8.3	to 48	13.3	to 49	18.3	to 82.3	25	to 123.3
9	to 37.7	13.3	to 51.7	18.7	to 100	25.7	to 104.7
9	to 47.7	13.7	to 53.3	19	to 97.3	26	to 110
9.3	to 43	13.7	to 54.3	19.3	to 79.7	27.3	to 125.7
9.3	to 46	13.7	to 65.7	19.7	to 98.7	27.7	to 124
10	to 53.3	14	to 70.3	19.7	to 100.7	28	to 128
11	to 50.7	15.3	to 68.7	20.3	to 109.3	30.3	to 132.3
11	to 57.7	15.3	to 69	20.7	to 87.3	31.7	to 140
11.3	to 68.3	15.3	to 74	20.7	to 90.3	33.3	to 127*
12	to 55.7	15.7	to 59.5	20.7	to 94.3	36	to 117.7*
12.3	to 48.3	16	to 65.3	20.7	to 109.3	37	to 112.7*
12.7	to 49.3	16	to 73.7	21.3	to 89.7	37	to 120*
12.7	to 58.7	16.3	to 89	22.7	to 112.3		
13	to 52.3	17.7	to 91.3	24	to 120.3		

* The u test values are greater than 1.96 and therefore the expected 5 x LC and observed HC counts are not members of the same distribtuion of means. The upper limit of the counting range for mK4 is 127.

DISCUSSION

Media Selection

Methods currently available to monitor surface waters for bacterial pollution have primarily been based on the concentrations of fecal coliforms (FC) or E. coli (EC). It has become apparent that analysis of pulp and paper mill effluent and waters significantly impacted by them yields inaccurate results when standard FC or EC procedures are used. The problem has been created by the presence of large concentrations of Klebsiella spp. in these waters that grow as target colonies on the FC/EC media. Since Klebsiella spp. are ubiquitous in nature (Huntley et al., 1976; Duncan and Razzell, 1972; Dufour and Cabelli, 1976; Caplenas et al., 1981; Hendry et al., 1982) high concentrations of this organism in natural waters can lead to a false impression of fecal pollution.

Several different types of media were used in this investigation in order to determine which was most suitable for isolation of Klebsiella spp. from natural and polluted water supplies.

Tables 3, 5 and 6 list the counts of target Klebsiella colonies on the media. Similar target colony counts were observed using mK2, mK4, mK5, mFC and mKleb. The other media were deemed unacceptable for quantification of Klebsiella for several reasons including difficulty in distinguishing target from background colonies and poor target colony recovery.

The media UK1 and UK2 (Table 3) had target colony counts approximately 100 fold greater than those listed for the other media. This may be attributed to inclusion of a large proportion of background colonies in the target colony counts. Target colony enumeration on UK1 and UK2 was difficult primarily because of the size and coloration of the colony. A

strong urease positive reaction was rarely observed using these urea based media. Instead, the predominate Klebsiella spp. colony morphology on UK1 and UK2 was 1 mm in size and a very light shade of rose. Presumptive target colonies recovered on these media were identified and only 50 - 60% of these colonies were identified as Klebsiella spp. Problems with the urease reaction may be due to the fact that urease production is at best only 86% reproducible among serial transfers of Klebsiella spp. (De Silva and Rubin, 1977). For these reasons, UK1 and UK2 are not suited to accurate quantification of Klebsiella spp. in surface waters.

The medium mCIK was found to be unsuitable for enumeration of Klebsiella in surface water samples. Problems were encountered regarding target colony description. Tomas and co-workers (1986) state that typical Klebsiella target colonies appeared as pink to red in color; size was not an important criterion. In our analyses, very few pink or red coloured colonies were observed. Most of the colonies on this medium were brown with an irregular edge. A representative number of the different colony types were picked from this medium and identified. Table 4 lists the speciation of these isolates. A large percentage of the colonies were found to be of the Pseudomonas group.

The lack of a clear cut colony type led to the modification of mCIK by the addition of 0.2 g/L of neutral red. This new medium, mCIK2 was tested for its ability to specifically recover Klebsiella pneumoniae; however, the same problems encountered using mCIK were noted using mCIK2.

This medium was also found to be extremely harsh to several different environmental strains of Klebsiella pneumoniae. Although in quality control analysis they displayed the typical target colony morphology, only a few colonies were observed along a heavy inoculum line.

A high interference of background colonies in the enumeration of target colonies on mCIK (Table 6) and mCIK2 coupled with the undistinguishing target colony morphology made this medium unsuitable for the isolation of Klebsiella pneumoniae. Dutka and co-workers (1987) have reported that underestimation of the total Klebsiella population and a 77% positive identification of presumptive target colonies makes this formulation of mCIK unacceptable for standard use.

Two other media which were deemed inappropriate for enumeration of Klebsiella pneumoniae were mK6 and mK6B. The first screening test using mK6 (Table 3) did not yield any results as colony growth was not observed. These two media, mK6 and mK6B, base their selectivity on the presence of a dye, methyl-violet 2B which is known to inhibit bacterial growth (Campbell and Roth, 1975). Campbell and co-workers (1976) state that this medium should easily differentiate Klebsiella pneumoniae from Enterobacter aerogenes on the basis of colony morphology.

Recovery of Klebsiella spp. on mK6 was not observed even using spiked samples of Klebsiella pneumoniae at concentrations of 10^8 per mL of sample water. The lack of growth on this medium was thought to be due to the high concentration of methyl-violet 2B. Therefore, the concentration of this basic dye was reduced to 0.2 g/L. This medium, mK6B, was found to support bacterial growth. Colonies were of a variety of sizes, shapes and consistancies. Typical target colony morphology observed by Campbell and Roth (1975) was 3 - 5 mm and pale to medium purple in colouration. Background colonies were found to be from less than 1 to 3 mm in size and tended to be darker purple in color. In this study, large glistening colonies were not observed. Instead, most of the colonies observed on mK6B typified background colonies described by Campbell and Roth (1975).

The medium mKleb2 was devised in hopes that it would be highly selective for Klebsiella spp. while providing results within a 24 h period. The theory was to have a medium which provided an abundance of nutrients, the selective agents of tellurite and carbenicillin and the visual identification of target colonies by the utilizatyion of indoxyl-β-D-glucoside.

Results showed that mKleb2 was somewhat successful at target colony recovery, however target colony counts were approximately 1 log unit lower than those listed for mFC, mK2, mK4, mK5 and mKleb (Table 6). A large proportion of the colonies were background types with numbers often equalling or surpassing target colony counts. Although this medium produced relatively easy identification of target colonies, the medium was not too selective.

The media most suited to isolation of Klebsiella spp. were mFC, mK2, mK4, mK5 and mKleb. These media were found to recover Klebsiella at similar rates and target colony morphology was easy to distinguish from background colonies.

mFC is a nutrient rich medium with the selective agent of carbenicillin (Niemela and Vaatanen, 1982). Target colonies appear as soft blue to bluish grey due to acid production from utilization of inositol (Figure 1). All presumptive target colonies of Klebsiella spp. produced a dark blue coloration of the medium directly under the colonies. All of these colonies were positively identified as Klebsiella spp. This characteristic may be of value when high background levels are present on the filter interfering with the counting of target colonies. Background colonies generally appeared as white, orange or yellow in color.

Target colonies were easiest to assess after 16 h of incubation on mFC although 24 h is recommended (Niemela and Vaatanen, 1982). At the 16 h time period, colonies were of a good size and bluish in coloration. Prolonged incubation of the plates made target colony counting more difficult due to overgrowth of the target and background colonies, especially in heavily contaminated water samples. The bluish tint of the target colonies faded to grey by the 24 h period.

The medium mK2 was suitable for recovering Klebsiella pneumoniae from water samples. Target colonies appeared as yellow mucoid and were 2 - 5 mm in diameter (Figure 2). Colonies were best enumerated at 40 h post incubation as after this time colonies developed an orange-red coloration. In addition, colonies were seen to overgrow and coalesce together.

One problem with this medium was that some strains of Pseudomonas were able to be recovered (Table 4). Colonies were 1 mm in diameter varying from white to yellow in color. As a result, target colony counts should not include yellow pinpoint colonies.

Target colonies recovered on mK4 were easy to distinguish from background colonies by the utilization of the specific carbohydrate indoxyl-β -D-glucoside. The incorporation of the carbohydrate made this medium highly effective for recognition of target colonies. A blue halo develops around the bacterial colonies that are able to utilize this carbohydrate (Figure 3). Klebsiella spp. appeared as 2 - 5 mm colonies grey in color surrounded by a blue halo. Those colonies which appeared as black centered with a blue halo were not considered as targets. Black centered colonies were identified as Pseudomonas spp.

Colony crowding did not appear to be a problem using this medium even after the 48 h incubation period. After the 24 h incubation period a blue

coloration was apparant where presumptive target colonies were to develop. This characteristic would be very useful as the plates could be checked at the 24 h period for the number of blue pinpoints observed on the filter. If there was too few or too many blue dots present on the medium, the corresponding water sample could be diluted accordingly and refiltered. This would serve to minimize the time for the reporting of results. It must be remembered however, that not all of the blue dots will develop as target colonies.

The presence of the chemical solution potassium tellurite in mK5 made the medium quite selective for Klebsiella spp. This group of organisms has a high degree of resistance to tellurite and this resistance is usually of chromosomal orgin (Tomas et al, 1984). Coupled with this chemical is the carbohydrate indoxyl- β-D-glucoside. Consequently, the target colonies appear as black/grey centered (due to the reduction of tellurite) with a blue halo (Figure 4). Colonies were found to average 2 - 4 mm in size.

This medium was found to have a lower total target and background bacterial count. Target colony counts generally averaged from .2 to .5 log units lower than those listed for mK2, mK4, mFC and mKleb (Tables 3, 4 and 6). However, background levels were less than observed on the other 4 media previously mentioned. This may be attributed to the presence of tellurite, making the medium more harsh.

The incorporation of the carbohydrate indoxyl- β-D-glucoside in this medium makes it attractive for two reasons: first, the association of a blue coloration around the target colonies; and second, the presence of this color occurs at the 24 h period. Therefore plates may be checked after 24 h incubation for any refiltering of a water sample that may be necessary.

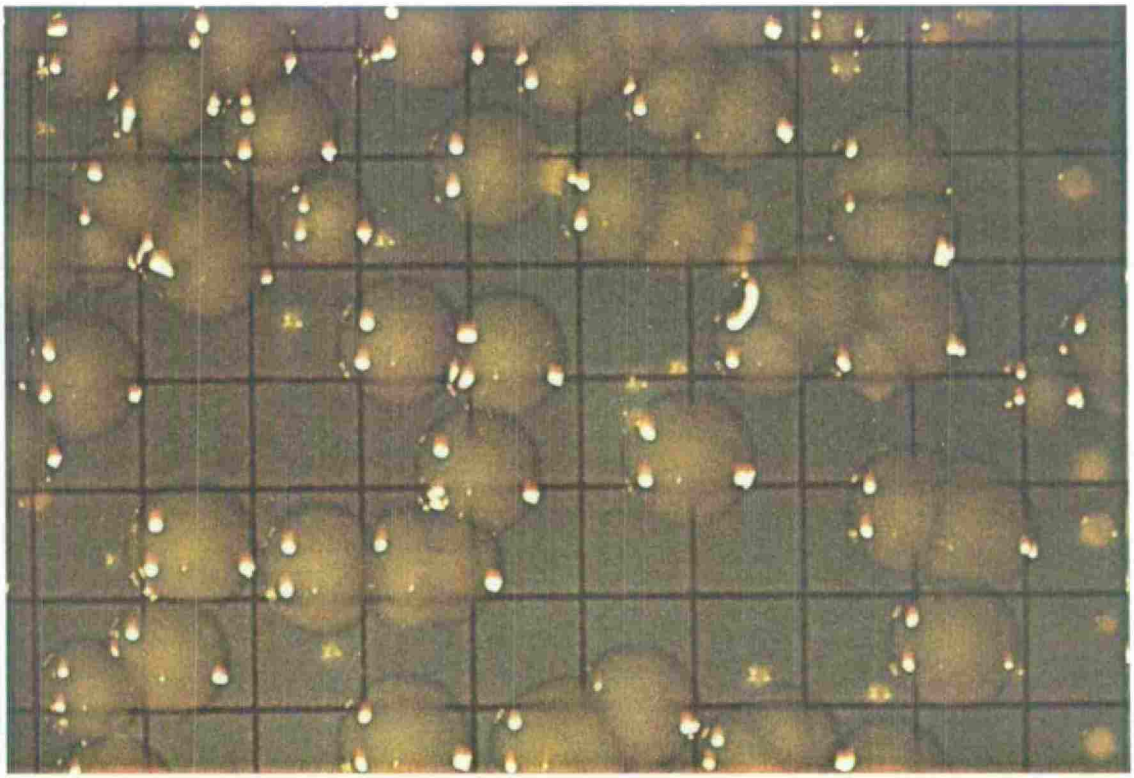

FIGURE 1: Target colonies appear as mucoid grey on mFC.

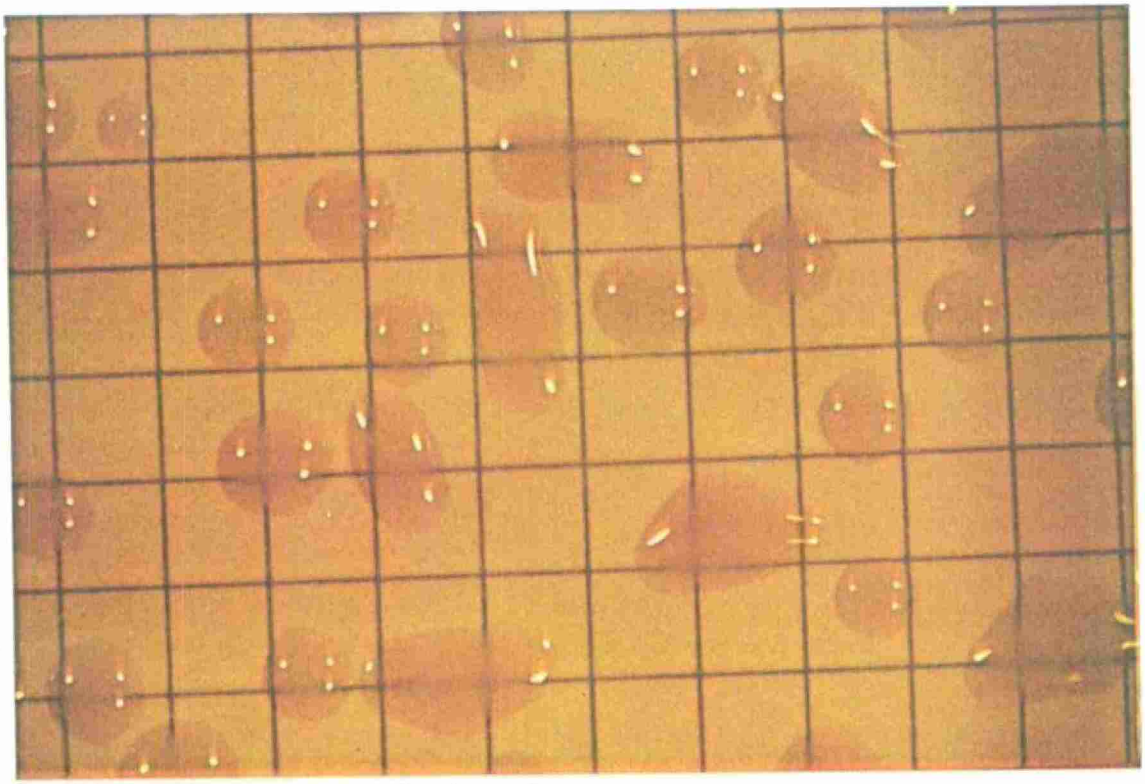

FIGURE 2: Target colonies observed on mK2.

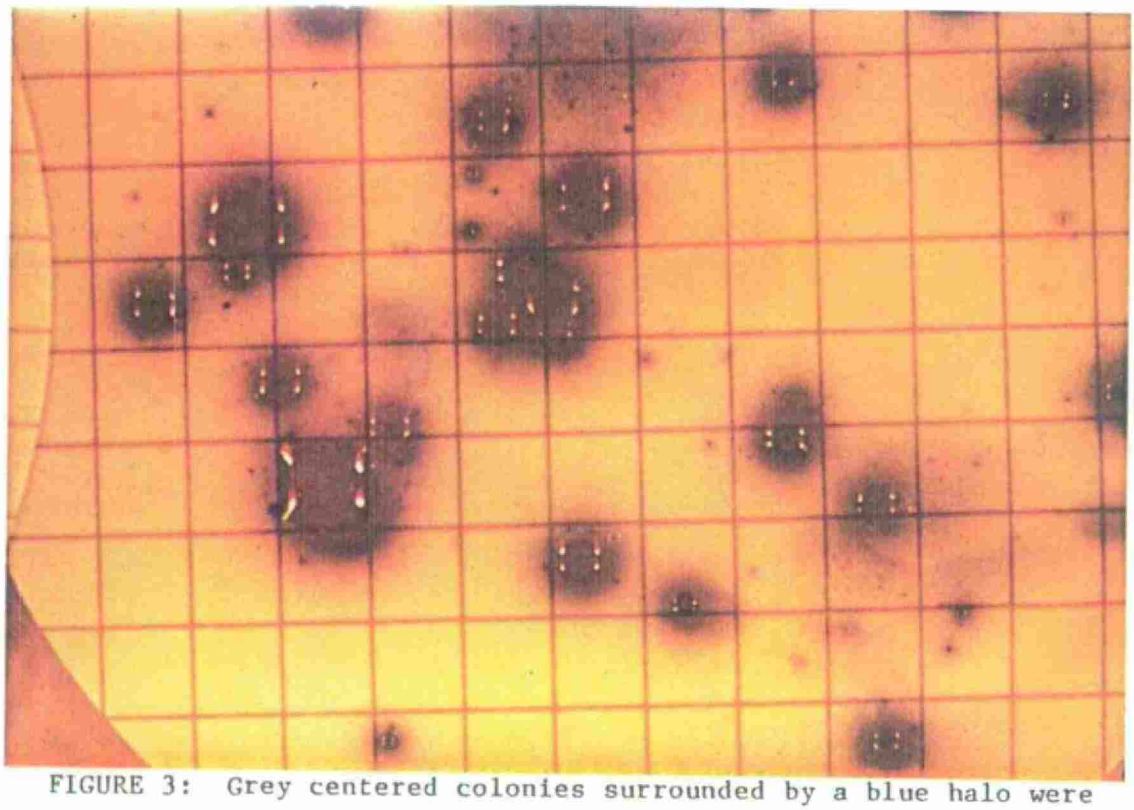

FIGURE 3: Grey centered colonies surrounded by a blue halo were regarded as target colonies on mK4.

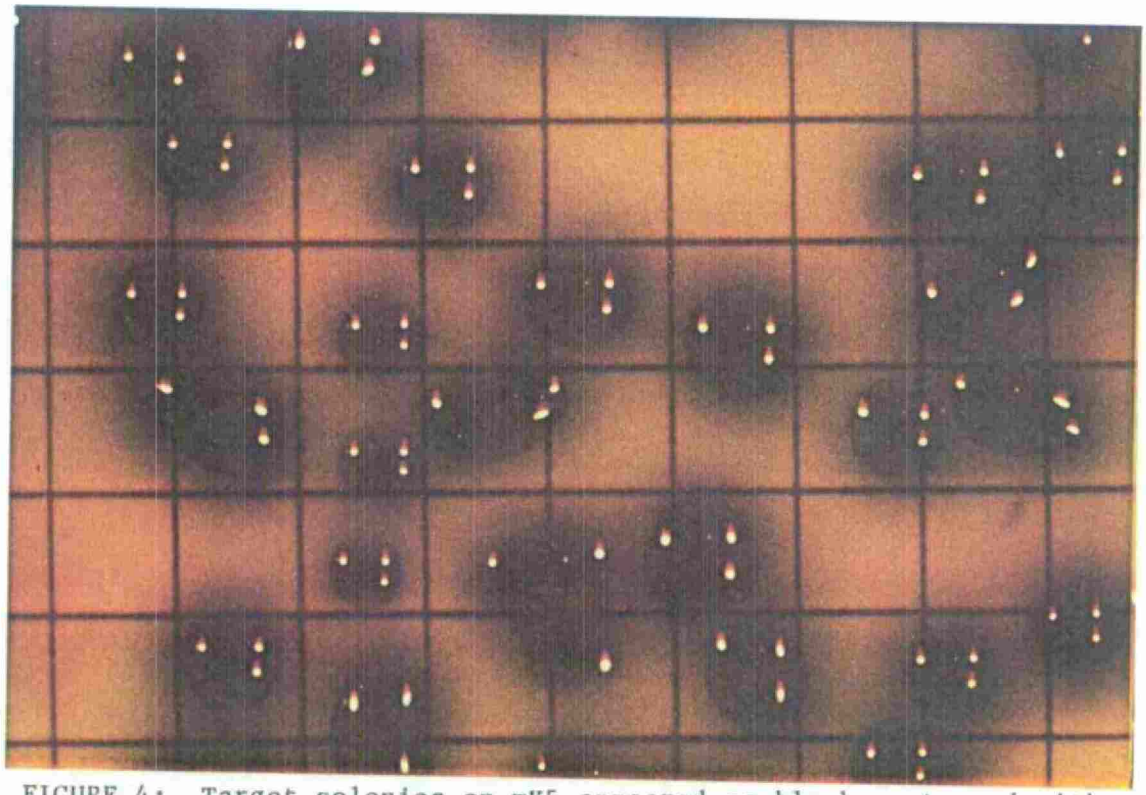

FIGURE 4: Target colonies on mK5 appeared as black centrered with a blue halo.

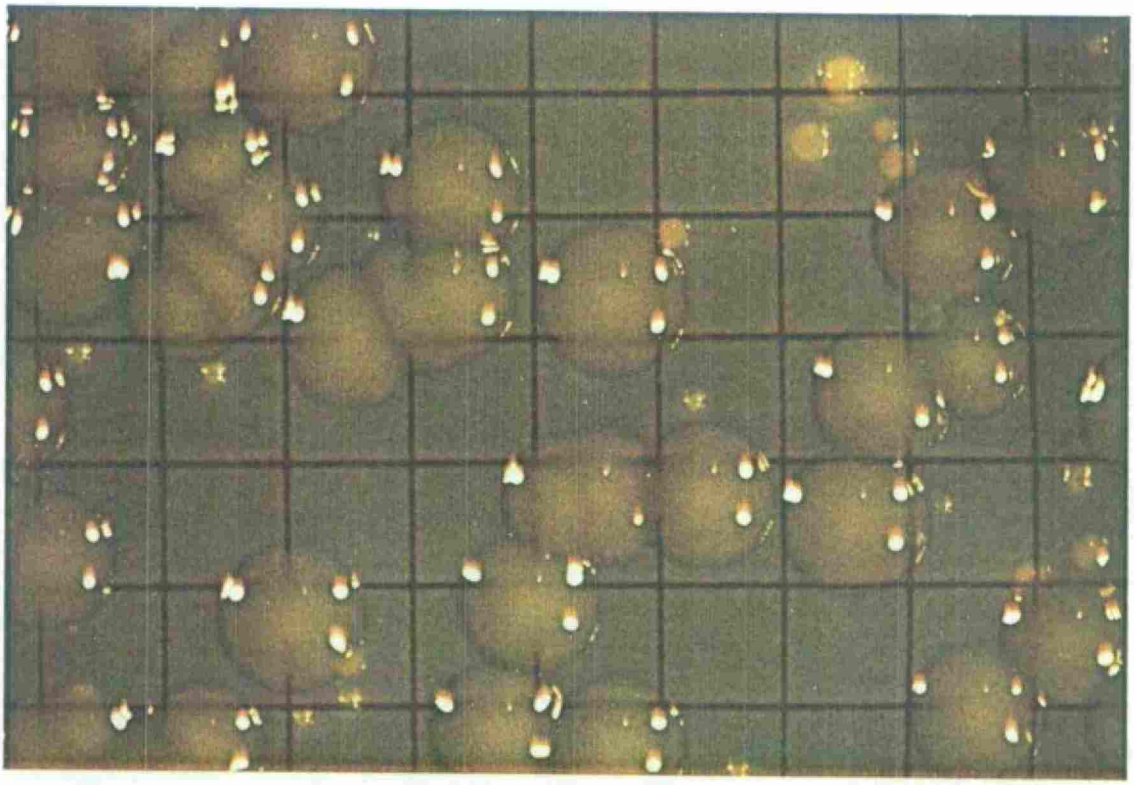

FIGURE 5: Grey and bluish grey colonies were considered as target colonies on mKleb.

The last medium which was selected to be analysed statistically was mKleb. Target colonies on this medium appeared as blue/grey in color while background colonies appeared as orange, white or yellow in color (Figure 5). The size of the Klebsiella target colonies ranged from 3 - 5 mm. The area of the target colony directly below the filter was found to be green/black in color. This characteristic was of use when dealing with high background levels on the filter.

Target colony counts on mKleb were best made at 18 h post incubation. Since mKleb is a nutrient rich medium, colonies have a tendency to overgrow with prolonged incubation. In addition, the colonies were found to lose their bluish tint after 20 h of incubation.

Identification of Target Organisms

Recent studies have shown that hospital acquired Klebsiella infections are not limited to the particular species and biotype Klebsiella pneumoniae sensu-stricto (Bagley, 1985; Woodward et al, 1979). In fact, a number of nosocomial and environmentally derived infections have been attributed to the different species of Klebsiella, namely K. pneumoniae non sensu-stricto and K. oxytoca (Bagley, 1985). For these reasons, it is important that in selecting a medium to quantitate Klebsiella from various sources, one should consider isolation of the two latter groups in conjunction with K. pneumoniae sensu-stricto.

The five media that were most suited to isolation of Klebsiella were mFC, mK2, mK4, mK5 and mKleb. Bacterial colonies that were considered as presumptive target colonies were picked from the filters, subcultured and subsequently identified. Of the 3190 isolates, approximately 93% were identified as members of the Klebsiella group (Table 7). The medium mK2 had

the highest positive identification of presumptive target colonies at 96% while the other four media had approximately 93% of the presumptive target colonies identified as Klebsiella spp.

Further breakdown of the isolates into their particular species revealed that 16% of the presumptive target colonies on each medium were identified as K. oxytoca. This organism is reported to be the second most commonly isolated Klebsiella spp. in nosocomial infections (Bagley and Seidler, 1977; Naemura and Seidler, 1978).

Nine percent of the presumptive target colonies on mK5 were K. pneumoniae sensu-stricto while approximately 17% of the presumptive colonies on mFC, mK2 and mK4 were K. pneumoniae sensu-stricto. The lower percentage of this particular biotype recovered on mK5 may not be due to the medium but due to the fact that only a portion of the presumptive target colonies were picked from this medium.

The use of the API 20E system in identification of the Klebsiella group has been found to be effective (De Silva and Rubin, 1977). Genus and species identification of Klebsiella pneumoniae has been found to be 100% reproducible; however, biotype reproducibility was only 64%.

The very high rate at which Klebsiella spp. were positively identified using these five different media suggests that each would be very useful in assessing levels present in the environment.

Specificity and Selectivity

The performance characteristic specificity refers to 'the ability of a method to select and distinguish the microorganism under consideration from all others in the same environment' (ASTM, 1986). Two separate values can

be obtained from the analysis: false positive error and the undetected target error.

Four of the media, mFC, mK2, mK5 and mKleb, had similar false positive error values of approximately 0.014. The medium mK4 had a slightly higher false positive error of 0.058. The closer this value is to zero, the more specific the method.

The term undetected target error refers to those colonies on the medium which do not appear as target colonies on the isolation medium. The media mK5 and mFC had undetected target errors of 0. The other media had undetected target errors of 0.015 for mK4, 0.043 for mK2 and 0.062 for mKleb. These low values suggest that very few of the background colonies on each of the media tested are in fact target colonies.

The index of selectivity refers to 'the ability of a method to encourage growth of target organisms while retarding development of non target organisms' (ASTM, 1986). The results (Table 8) show that mK2, mK4, mK5 and mFC were very similar with respect to the index of selectivity; values calculated to be approximately 0.64. The medium mKleb had the lowest index of selectivity of 0.53. These results indicate that mKleb is the least selective of all of the five media tested.

Based on the above information, the three media with the two best performance characteristics are mK4, mK2 and mFC. The medium mK4 was selected over mK5 due to the previously determined recovery counts of presumptive target colonies. mK4 had higher presumptive target colonies by approximately 0.4 log units over that of mK5 (Tables 3, 5 and 6). This fact coupled with the very similar selectivity and specificity values lead to the choice of mK4 over mK5.

Accuracy and Upper Counting Range

Accuracy is defined as 'the degree of agreement between the density of microbe obtained with a test method and the density obtained with an acceptable reference method' (ASTM, 1986). The reference medium chosen was mK base. This medium has the same nutrients as mK2, while omitting the selective chemical and antibiotic agents.

The accuracy at 0 h for mFC, mK2 and mK4 were essentially equal, values were calculated to be 114.5% recovery compared to the reference method. After stressing the cultures for 24 h at 10°C, the accuracy of the media was found to differ. mK2 had the highest 24 h accuracy, 118%, followed by mK4 at 111% and finally mFC at 81.9% (Table 9). These results indicate that mFC is not able to recover stressed cultures of Klebsiella spp. as well as the other two media.

The upper counting limit refers to 'that point above which the reliability of the colony count on a single plate or membrane from a specified volume is affected by uncontrollable factors' (ASTM, 1986). Uncontrollable factors include crowding, nutrient limitation and background interference. The three media tested were found to have different upper counting limits. Two of the media, mFC and mK4 had upper counting limits of 127 colonies per filter, while mK2 had an upper counting limit of 115 colonies.

The lower counting limit of mK2 may be attributed to the formation and morphology of the colonies in heavily contaminated samples. When total presumptive target colonies exceeded 100 on mK2, colonies coalesced together (Figure 2) and in many cases one very large colony was observed. As a result, an underestimation of the total presumptive target colonies on several areas of the filter may occur. In comparison, presumptive target

colonies on mK4 did merge together, however they did not form a large uniform colony. Separate colonies were usually distinguishable in a large cluster by virtue of the colony centre and colony edge (Figure 1).

SUMMARY

The two media most suited to recovery and determination of Klebsiella spp. are mK2 and mK4. Greater than 93% of the presumptive target colonies were identified as Klebsiella spp, accuracy values at 0 and 24 h were greater than 110% compared to the recovery medium mK base, and the specificy and selectivity values were comparable. mK4 had a higher counting range, 127 colonies, compared to that of mK2, 115 colonies.

Comparing these two media on a laboratory performance level, mK4 is the preferred medium for several reasons.

1) Presumptive target colonies were extremely easy to distinguish from background colonies on mK4. This was due to the incorporation of indoxyl-β-D-glucoside in the medium. Presumptive target colonies have a blue halo surrounding a grayish-blue centered colony.

2) Colony crowding was not a problem on mK4, further proven by the higher upper counting range. In addition, after prolonged incubation if colonies did coalesce the number of target colonies in a cluster was discernable due to the shape of the blue halo and due to the fact that these colonies did not flow together as seen on mK2.

3) Prolonged incubation on mK2 hampered the target colony enumeration because colonies lost their typical yellow coloration.

4) Pseudomonas spp. appeared as small target colonies on mK2.

5) An estimation of the Klebsiella population could be made on mK4 at 24 h post incubation, by observation of the blue dots on the filter. If the correct dilution for filtering was missed, the corresponding sample could be refiltered, hence minimizing the time period for the reporting of results (reliability of bacterial levels in water samples decreases after 24 h).

The only drawback of using mK4 is its expense. Currently indoxyl- β - D-glucoside costs approximately $ 200.00 per gram. Therefore, production of mK4 plates is about $1.00 more per plate over that of mK2. However, the five above mentioned points far outweigh the financial aspects of this medium.

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APPENDIX

Media Ingredients

mK2

Inositol	4.0 g
NaCl	1.0 g
KCl	4.0 g
K_2HPO_4	1.5 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.1 g
Agar	15.0 g
dH_2O	760 mL

- Mix and bring to boil (90°C). Autoclave 15 min, 15 psi at 121°C.
- Cool to 55°C and add uric acid and inhibitor solutions.
- Add 20 mL of ethanol, mix well and dispense.
- Final pH 7.35 $\pm$ 0.2.

Uric acid solution: Mix 0.3 g of uric acid in 200 mL of sterile distilled water. After mixing, add 1N NaOH to bring uric acid into solution. Adjust final pH to 8.0 with 1N HCl. Filter sterilize.

Inhibitor solution: Mix 0.1 g of phenol red, 0.4 g sodium taurocholate and 0.05 g of carbenicillin in 41 mL of sterile distilled water.

mK4

- Same instructions for mK2 but omit phenol red.
- Add 0.25 g/L indoxyl-β-D-glucoside (dissolved in 5 mL ethanol) after autoclaving.

mK5

- Same instructions for mK2 but omit phenol red.
- Add 0.25 g/L indoxyl-β-D-glucoside (dissolved in 5 mL ethanol) and 1 mg/L K_2TeO_3 (dissolved in 5 mL dH_2O). Filter sterilize each solution separately before adding to autoclaved medium (55°C).

mK6

- Same instructions for mK2.
- Add 2 g/L methyl violet 2B prior to autoclaving.

mK6B

- Same instructions for mK6 but decrease concentration of methyl violet 2B to 0.2 g/L.

mKleb

Phenol red agar base	31.0 g
Inositol	5.0 g
Aniline blue	0.1 g
Sodium lauryl sulphate	0.1 g
dH_2O	1.0 L

- Heat above ingredients to dissolve. Autoclave 15 min at 121°C.
- Cool to 55°C.
- Add 0.05 g carbenicillin and 20 mL of 95% ethanol.
- Mix and dispense.
- Final pH 7.2 ± 0.2.

mKleb 2

Bacto beef extract	1.0 g
Proteose peptone #3	10.0 g
Sodium chloride	5.0 g
Inositol	5.0 g
Sodium lauryl sulphate	0.1 g
Agar	15.0 g
dH_2O	1.0 L

- Heat to dissolve. Autoclave 15 min at 15 psi, 121°C.
- Cool to 55°C. Add tellurite and carbohydrate solutions.
- Final pH 7.2 ± 0.2.

Tellurite solution: Dissolve 1 mg K_2TeO_3 and 0.05 g of carbenicillin in 10 mL of distilled water. Filter sterilize.

Carbohydrate solution: Dissolve 0.25 g indoxyl- β-D-glucoside in 20 mL ethanol. Filter sterilize.

mCIK

Proteose peptone #3	5.0 g
Yeast extract	3.0 g
NaCl	7.5 g
K_2HPO_4	3.3 g
KH_2PO_4	1.0 g
Sodium lauryl sulphate	0.2 g
Sodium deoxycholate	0.1 g
Agar	15.0 g
dH20	1.0 L

- Heat to dissolve. Autoclave 15 min at 121°C, 15 psi.
- Cool to 55°C, add 3 mg filter sterilized K_2TeO_3 (3 mg dissolved in 5 mL dH_2O) and 4 g filter sterilized inositol solution (dissolved in 20 mL dH_2O).
- Mix well and dispense. Final pH 7.1 ± 0.2.

mCIK2

- Same instructions as listed for mCIK but add 0.02 g/L of neutral red prior to autoclaving.

Modified mFC Agar

- Use sterile glassware.

Tryptose	10.0 g
Proteose peptone #3	5.0 g
Yeast extract	3.0 g
Inositol	12.5 g
Sodium deoxycholate	0.1 g
Sodium lauryl sulphate	0.2 g
NaCl	5.0 g
Aniline blue	0.1 g
Agar	15.0 g
Sterile dH_2O	1.0 L

- Heat to boiling to dissolve. Add 10 mL of a 1% solution of Rosolic acid. Continue heating for 1 min.
- Cool to 55°C and add 0.05 g carbenicillin. Mix and dispense.
- Final ph 7.4 $\pm$ 0.1.

UK1

Urea agar base	29 g
dH_2O	100 mL

- Dissolve base in water and filter serilize.

Agar	15 g
dH_2O	900 mL

- Mix, heat to dissolve and autoclave 15 min at 121°C, 15 psi.
- Cool agar base to 55°C, add urea solution and 0.05 g/L carbenicillin.
- Allow to mix and pour into dishes.
- Final pH 6.9 $\pm$ 0.1.

UK2

- Same instructions as for UK1.
- Add 0.2 g/L indoxyl-β-D-glucoside (dissolved in 5 mL ethanol) after autoclaving.

mK Base

- Same instructions as for mK2 but omit uric acid and inhibitor solutions.

40% Glycerol Solution

- Dissolve 45 g Tri-sodium citrate in 900 mL dH_2O. Add 600 mL of glycerol and dispense into 16 x 100 mL tubes, 1 mL per tube.
- Autoclave 15 min, 15 psi at 121°C.

Sodium Thiosulphate Solution

$Na_2S_2O_3.5H_2O$	62.5 g
dH_2O	500.0 mL

- Add 0.2 mL of this solution to each 8 oz. sample bottles to achieve a final concentration of 100 ppm.
- Autoclave bottles for 20 min at 121°C.

Oxidase Reagent

NNNN Tetramethyl-p-phenylenediamine dihydrochloride	0.25 g
Ascorbic acid	0.025 g
Sterile dH_2O	25 mL

- Add ascorbic acid to sterile water to give a 0.1% solution.
- Add remaining chemical and bring into solution.
- Store at 4°C in dark bottles.

Sorbose Broth

Phenol red broth base	16 g
dH_2O	900 ml

- Bring broth base into solution and autoclave for 15 min at 121°C.
- Cool to 50°C and add filter sterilized sorbose solution (5 g dissolved in 100 mL of sterile dH_2O).

Hydroxy L-Proline Broth

Solution A:	
Tris (hydroxymethyl)aminomethane-HCl	10.0 g
Ammonium chloride	2.0 g
Potassium chloride	2.0 g
KH_2PO_4	1.0 g
Na_2SO_4	0.1 g
dH_2O	900.0 mL
Solution B:	
$CaCl_2.2H_2O$	0.05 g
$MgCl_2$	0.02 g
dH_2O	100.00 mL
Solution C:	
Hydroxy L-proline	1.0 g
dH_2O	100.0 mL

- Autoclave solutions A and B separately. Filter sterilize solution C.
- To make up final broth, mix 90 mL of A, 1 mL of B and 10 mL of C.
- Final pH 7.0.

Buffered Dilution Blanks

- Dissolve 38 g of $MgCl_2$ in 1 L of distilled water.
- Autoclave at 121°C for 15 min.
- Use 1.25 mL of stock phosphate buffer and 5 mL of stock magnesium chloride per litre of distilled water.
- Fill dilution blanks and autoclave for 15 min at 121°C.
- Store at 4°C.

Phosphate Buffer Solution

- Dissolve 34 g of KH_2PO_4 in 500 mL distilled water.
- Adjust pH 7.2 $\pm$ 0.2 with 1 N NaOH.
- Fill up to 1 L mark with distilled water.
- Autoclave for 15 min at 121°C.
- Store at 4°C for up to 1 month.
- Dispense 1.25 mL per litre of distilled water.

	DATE DUE		